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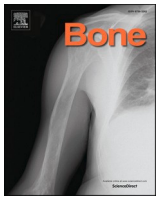

Full Length Article

The effects of endurance trainability phenotype, sex, and interval running training on bone collagen synthesis in adult rats

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ABSTRACT

Bone is influenced by many factors such as genetics and mechanical loading, but the short-term physiological effects of these factors on bone (re)modelling are not well characterised. This study investigated the effects of endurance trainability phenotype, sex, and interval running training (7-week intervention) on bone collagen formation in rats using a deuterium oxide stable isotope tracer method. Bone samples of the femur diaphysis, proximal tibia, mid-shaft tibia, and distal tibia were collected after necropsy from forty-six 9 ± 3-month male and female rats selectively bred for yielding low (LRT) or high (HRT) responses to endurance training. Bone collagen proteins were isolated and hydrolysed, and fractional synthetic rates (FSRs) were determined by the incorporation of deuterium into protein-bound alanine via GC-pyrolysis-IRMS. There was a significant large main effect of phenotype at the femur site ($p < 0.001$; $\eta_g^2 = 0.473$) with HRT rats showing greater bone collagen FSRs than LRT rats. There was a significant large main effect of phenotype ($p = 0.008$; $\eta_g^2 = 0.178$) and a significant large main effect of sex ($p = 0.005$; $\eta_g^2 = 0.196$) at the proximal site of the tibia with HRT rats showing greater bone collagen FSRs than LRT rats, and male rats showing greater bone collagen FSRs compared to female rats. There was a significant large main effect of training at the mid-shaft site of the tibia ($p = 0.012$; $\eta_g^2 = 0.159$), with rats that underwent interval running training having greater bone collagen FSRs than control rats. Similarly, there was a significant large main effect of training at the distal site of the tibia ($p = 0.050$; $\eta_g^2 = 0.156$), with rats in the interval running training group having greater bone collagen FSRs compared to rats in the control group. Collectively, this evidence highlights that bone responses to physiological effects are site-specific, indicating that interval running training has positive effects on bone collagen synthesis at the tibial mid-shaft and distal sites, whilst genetic factors affect bone collagen synthesis at the femur diaphysis (phenotype) and proximal tibia (phenotype and sex) in rats.

1. Introduction

Bone is a dynamic tissue that is constantly (re)modelling. Throughout life, the skeleton is "constructed" and "reconstructed" by the physiological processes of bone modelling and remodelling, which control bone mass [1]. Understanding the short-term physiology of bone

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and how it is influenced by different factors (e.g., heritability, age, sex, lifestyle) is vital for the development of interventions that can preserve and improve bone health.

The genetics are an essential determinant of bone mass, typically referred to as bone mineral density (BMD). Twin and family studies reported that the heritability of BMD ranged between 50 and 85 %, depending on the investigated skeletal site and population [2]. Genome-wide association studies have identified various genes that influence skeletal traits (i.e., BMD and osteoporosis risk), which again are different at various skeletal sites [3]. Gene-environment, and particularly gene-physical activity, interactions may account for some of the unexplained heritability [4] and explain the inter-individual differences in BMD across lifespan [5]. Clark and Duncan [6] proposed that "extreme cohorts" can present genetic variants that have stronger, easier to identify, associations with relevant phenotypes. For example, it has been suggested that elite athletic cohorts, who experience high mechanical stress and are at the extremes of human endurance capacity, and who usually present high BMD at loaded skeletal sites and, might possess a genotype exceptionally suitable to tolerate those stresses [5]. How this potential genotype-phenotype relationship translates into differences in the active process of bone (re)modelling (e.g., higher bone formation), is unknown; but would provide a basis to the hypothetical phenotypical adaptation to large training volumes and mechanical stress. The selectively bred animal model of low and high responders to endurance training generated by Koch & Britton [7] is a useful tool for studying these "extreme cohorts" and the potential benefits on bone health with acquired endurance exercise capacity. Furthermore, the acquisition of bone mass, quantitatively, follows age and sex specific patterns [8]. In terms of sex differences, human and animal males have generally greater bone mass (i.e., BMD) compared to females; a difference that surfaces as sexual maturation progresses [9]. Sex steroid hormones (e.g., oestrogens and androgens) promote the acquisition of bone mass during puberty and are responsible for the differences between the female and male skeleton [10]. Although sex steroids affect both bone-resorbing osteoclasts and bone-forming osteoblasts [9], their direct impact on short-term bone (re)modelling is yet to be determined.

Exercise has long been regarded as a fundamental lifestyle factor that affects bone (re)modelling, given the positive relationship between mechanical loading and the skeleton [11]. As such, exercise interventions consisting of weight bearing activities are associated with long-term improvements in BMD, particularly at the load bearing sites [12–16]. The best exercise regimen (i.e., type, intensity, duration, and frequency) to optimise bone osteogenic responses, however, is still not well defined; and the bone responses to some activities, such as running exercise, are not clear. Whilst running produces gravitational loading, it presents a repetitive loading cycle and the beneficial effects of mechanical loading may not counteract the potential negative influences associated with endurance exercise [17], such as micro-damage accumulation, high prevalence of stress fracture injury and low nutrient and/or energy availability [18].

How those factors and interventions affect bone (re)modelling in humans and animals remains unclear, partly because of the lack of methods that can directly measure dynamic and acute changes of bone formation and resorption. We have recently developed a deuterium oxide stable isotope tracer method for the quantification of bone collagen synthesis rates in vivo [19]. This method allows the direct measurement of the formation of newly synthesised bone matrix across days/weeks during the bone (re)modelling cycle at specific bone sites. Osteoblasts synthesise and secrete collagen to the bone extracellular matrix [20], which is then mineralised by the deposition of hydroxyapatite crystals containing phosphate and calcium among collagen fibrils forming new bone matrix [21].

The aim of this study was to investigate the differences in bone collagen synthesis between (i) low and high responders to endurance training, (ii) males and females, and (iii) trained with interval running and control adult rats using our deuterium oxide stable isotope tracer method. These differences were analysed separately in the femur diaphysis, tibial proximal metaphysis-epiphysis, tibial mid-shaft diaphysis, and tibial distal metaphysis-epiphysis sites. To explore the regulatory and signalling pathways that could explain those differences, a secondary aim was to evaluate expression of genes involved in bone (re) modelling in the tibial diaphysis.

All bone samples analysed herein are part of a secondary analysis performed on samples obtained from a prior study primarily designed to investigate exercise responses following interval running training across sex and phenotypes, using the selectively bred rat model inheriting either a low (LRT) or high (HRT) adaptive response to endurance training [7]. This prior study was not designed to directly maximise osteogenic responses.

2. Methods

2.1. Animals and intervention

All experiments were approved by the Animal Care and Use Committee of Southern Finland, license number ESAVI-2010-07989/Ym-23, STH 534 A (21.9.2010) and complements ESAVI/1968/04.10.03/2011, PH308A (30.3.2011) and ESAVI/722/04.10.07/2013, PH275A (1.3.2013); and were conducted in accordance with the Guidelines of the European Community Council Directive 86/609/EEC. Femur and tibia bones were obtained from forty-six 9 ± 3-month male and female rats from generations 17 and 18 of the LRT and HRT model [7] (Table 1). Briefly, using artificial selective breeding for >15 generations, a heterogenous N/NIH stock rat population was developed into two contrasting phenotypes yielding low (LRT) and high (HRT) responses to endurance training. This selection was defined on the magnitude of change in running capacity (quantified by maximal treadmill distance as a result of an 8-week endurance treadmill training protocol). After fifteen generations of selection, rats bred as HRT increased maximal treadmill running distance from 646 to 869 m (change, 223 ± 20 m), whereas LRT rats decreased from 620 to 555 m (change, −65 ± 15 m) after completing the same training [7]. The same animals and derived samples were used to develop a collagen extraction and deuterium oxide stable isotope tracer method for the quantification of bone collagen synthesis rates [19].

Rats were single housed in air-conditioned rooms at an ambient temperature of 21 ± 2 °C and relative humidity at 50 ± 10 %. Artificial lighting provided light cycles of 12:12-h light-total darkness. Commercially available pelleted rodent diet (R36; Labfor; Lantmän nen, Malmö, Sweden) and tap water (from the municipal water system of Jyväskylä, Finland) were available ad libitum. The energy content of the feed was 1260 kJ·100 g^{-1} (301.15 kcal·100 g^{-1}). The feed contained 18.5 % raw protein, 4.0 % raw fat, 55.7 % nitrogen-free extracts, 3.5 % fibre, 6.3 % ash, and 12 % water.

Rats of both low and high response phenotypes were first tested for their maximal running capacity according to the protocol described in Koch et al. [7], where rats completed three maximal running tests on a treadmill with 1-day rest in between, and the best result out of the three tests was considered for the determination of maximum speed. Then, during a 7-week intervention period, rats were divided into trained and control groups (Table 1), where rats in the trained groups underwent high-intensity interval running training on a treadmill. This 7-week long intervention was designed to investigate physiological training responses in this rat model. Interval running training consisted of a warm-up for 5 min at constant speed 9 m/min, followed by a 15 min session that comprised 3 × 3 min bouts of running at 85–90 % of individually determined maximum speed with 2-min recovery periods between each bout at ~50 % maximum speed with 15° uphill inclination. Training was completed three times per week with 1-day rest in between. Every two weeks one maximal running capacity test replaced a training session; and based on the result of the test, training speeds were adjusted individually for each rat. Overall, across the 7 weeks, rats in the trained

Table 1
Total number of femur and tibia bones available for analysis divided into phenotypes of low (LRT) and high (HRT) responders to endurance training, male and female, and control and interval running training groups.

	High responders to training (HRT)				Low responders to training (LRT)			
	Interval running training		Control		Interval running training		Control	
	Male	Female	Male	Female	Male	Female	Male	Female
Femur	$n = 6$	$n = 6$	$n = 5$	$n = 4$	$n = 5$	$n = 6$	$n = 4$	$n = 4$
Tibia	$n = 6$	$n = 7$	$n = 5$	$n = 5$	$n = 7$	$n = 6$	$n = 4$	$n = 5$

group completed treadmill running covering a total distance of 8980 ± 1460 m. During the 7-week intervention period, rats in the control groups were kept in the same conditions as the exercising rats but were not trained on the treadmill, and at the end of the intervention undertook three maximal running capacity tests. Throughout the last 3 weeks of the 7-week intervention period all rats received one gavage of 7.2 mL·kg BW^{-1} 70 % D_2O and thereafter drinking water was enriched to 2 % D_2O to maintain body-water enrichment. Necropsies were done ~48 h after the last maximal running test.

2.2. Blood and bone sample collection

Blood samples (~5 mL) were collected at necropsy and plasma was separated by centrifugation and stored frozen at -20 °C until analysis. Non-enriched blood was collected from a group of rats that did not receive D_2O for the determination of background enrichment. Left femur and tibia bones were rapidly exposed after necropsy, removed, and immediately frozen by complete immersion in liquid nitrogen and were kept frozen at -70 °C until analysis. Bone samples were obtained from the femur diaphysis (FEM), tibial proximal epiphysis-metaphysis (T-PRO), tibial mid-shaft diaphysis (T-MID), and the tibial distal epiphysis-metaphysis (T-DIS) as described in Civil et al. [19].

2.3. Isolation and derivatisation of bone collagen protein

Bone samples were demineralised, and collagen proteins were isolated and then hydrolysed to free amino acids. Briefly, bone samples were transferred into 0.5 M HCl solution for ~13 days or until samples were completely decalcified and appeared translucent and flexible. The HCl solution was changed for a fresh solution every 2–3 days with bouts of vortexing before and after each change, removing the mineral component dissolved in the solution. Any visually remaining connective tissue was manually removed with a sharp scalpel before or during the demineralisation process. Following demineralisation, bone samples were transferred to 0.3 M NaOH to dissolve and remove the remaining bone marrow and soluble proteins, leaving the bone collagen proteins. The NaOH solution was changed ~3 or more times over 5 days, or until it visually appeared that the bone marrow was completely removed, with bouts of vortexing and centrifuging before and after each change. Bone collagen proteins were hydrolysed to free amino acids by incubating in 0.1 M HCl in Dowex H+ resin slurry overnight at 110 °C before being eluted from the resin with 2 M NH4OH and evaporated to dryness. Amino acids were then derivatised as their N-methoxycarbonyl methyl esters as previously described [19].

2.4. GC–MS/MS body water enrichment analyses

Body water enrichment was measured in plasma by incubating 100 μL of each sample with 2 μL of 10 M NaOH and 1 μL of acetone for 24 h at room temperature. Following incubation, the acetone was extracted into 200 μL of n-heptane and 0.5 μL of the heptane phase was injected into a TRACE 1310 Gas Chromatograph connected to TSQ 8000 triple quadrupole GC–MS/MS (Thermo Scientific) for analysis. A standard curve of known D_2O enrichment was run alongside the samples for calculation of enrichment.

2.5. GC-pyrolysis-IRMS deuterated alanine analysis and calculation of fractional synthetic rates

Using protein-bound alanine offers a major advantage using D_2O methods to quantify protein synthesis [22,23]. Protein-bound alanine enrichment was determined by pyrolysis-gas chromatography with isotope-ratio mass spectrometry (Delta V Advantage, Thermo Scientific). Bone collagen fractional synthetic rate (FSR) was calculated from the incorporation of deuterium-labelled alanine into protein using the enrichment of body water, corrected for the mean number of deuterium moieties incorporated per alanine (3.7) and the dilution from the total number of hydrogens in the derivative (i.e., 11), as the surrogate precursor labelling over the 3-week period of D_2O labelling. The equation used was:

$$\mathrm{FSR} = -ln\left[\frac{1-\left(\frac{APEala}{APEp}\right)}{t}\right]$$

where APEala equals deuterium enrichment of protein-bound alanine, APEp indicates mean precursor enrichment over the time period, and t represents time (i.e., 21 days).

2.6. Gene expression analysis

RNA was extracted from bone samples of the tibia-diaphysis ($n = 39$) obtained with pestle and mortar (~23 g) and free of bone marrow. Frozen samples in nuclease-free tubes kept on ice were homogenised in 400 μL of TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) for 5 min, followed by three bouts of 30 s vigorous shaking in a TissueLyser II (Qiagen, Hilden, Germany) combined with 2 min on ice to avoid samples reaching a high temperature causing RNA degradation. To achieve phase separation 80 μL of chloroform was added into each tube, then vortexed for 30 s, and left to incubate for 5 min. Samples were then centrifuged at 17,000 *g* for 15 min at 4 °C. The RNA phase was then removed and mixed with an equal volume of 2-propanol, 1 μL of GlycoBlue (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was added and tubes were left on ice for 30 min before subsequent centrifugation at 17,000 *g* for 10 min at 4 °C. RNA pellets were washed with 70 % ethanol and centrifuged at 17,000 *g* for 1 min at 4 °C three times. Ethanol was removed and the pellet was left to air dry for 5 min and then resuspended in nuclease-free water. RNA concentration and purity of all samples was determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was diluted to 50 ng·μL^{-1} and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) following manufacturer instructions. The resultant cDNA was diluted to 10 ng·μL^{-1} before reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Real time RT-qPCR analyses were performed in triplicate using Sigma-Aldrich custom designed primers (Sigma-Aldrich, St Louis, MO, USA) for all genes (Table 2) and Power Up SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). In a 384-well PCR plate, 1 μL of diluted cDNA was added to each well containing the appropriate primers and Master Mix for a 7 μL total reaction volume.

Table 2
List of genes and primer sequences.

Gene	Primer sequency
B2M (beta-2-Microglobulin)	Fwd: CGGGGTGGTGATGAGAAGTT Rev.: AAGGCTCCTTGTCCCTTGAC
Bglap (osteocalcin)	Fwd: GTTTGAGGGGCCTGGGATTG Rev.: ACACAACTGCAGGTCGAGTTT
COL1A1 (collagen type 1 alpha 1)	Fwd: GTACATCAGCCCAAACCCCA Rev.: CAGGATCGGAACCTTCGCTT
COL1A2 (collagen type 1 alpha 2)	Fwd: GGGGTTGATGCAGACAGTCA Rev.: CCCACTCACTGCACATCACT
COL5A1 (collagen type 1 alpha 2)	Fwd: CCCAAAGAAAACCCAGGTTCC Rev.: CACAGGGTTGCCTTCAGCAT
IBSP (integrin-binding sialoprotein)	Fwd: GCCACACTCTCAGGGGTAAC Rev.: TGCATCTCCAGCCTTCTTGG
TNFRSF11B/OPG (osteoprotegerin)	Fwd: TGCTCCTGGCACCTACCTAA Rev.: GCACTCCTGTTTCACGGTCT
RANK (receptor activator of nuclear factor-kappa B)	Fwd: GCTACCACTGGAACGCAGACT Rev.: CGTTGAGCTGCAAGGGATGTT
RANKL (receptor activator of nuclear factor-kappa B ligand)	Fwd: GTCCAGGTGTCCAACCCTTC Rev.: CCATGCTAAGGCTCCACAAA
Runx2 (runt-related transcription factor 2)	Fwd: CGCCTCACAAACAACCACAG Rev.: AATGACTCGGTTGGTCTCGG
SOST (sclerostin)	Fwd: CAACCAGACCATGAACCGGG Rev.: TGTACTCGGACACGTCTTTGG
TGF-β (transforming growth factor beta)	Fwd: CAGTGCTGAGGAGAAACCGT Rev.: GCTCTCCATTGTCCCAGGTC
Wnt16 (protein Wnt-16)	Fwd: AGCATGACCGATGTCCACAC Rev.: AACACTCTTACAGGCAGCGA

The qPCR reaction was run using the following cycling conditions: an initial hold stage at 95 °C for 20 s, then 40 cycles of 95 °C for 1 s and 60 °C for 20 s (1.6 °C/s ramp rate), with a final melt curve stage of 95 °C for 15 s, 60 °C for 60 s; finishing at 95 °C for 15 s.

2.7. Statistical analyses

Descriptive statistics were performed for data sets to check for normal distribution (accepted if $p > 0.05$) using the Shapiro-Wilk test and homogeneity of variances by the Levene test (accepted if $p > 0.050$). The presence of extreme outliers was determined by the Rosner's test.

For the bone collagen FSR analysis, each bone site was considered as an independent data set with potential independent effects because we have previously reported differences between collagen FSR across bone sites [19]. Data sets for the FEM, T-MID and the T-DIS were log based (log10) transformed to achieve normal distribution and homogeneity of variances prior to analysis. Differences in bone collagen FSRs at the FEM, T-PRO, and T-MID sites were determined using three-way (phenotype x sex x training effect) ANOVA tests. The same analysis was conducted at the T-DIS site using a Robust three-way ANOVA test due to the presence of one outlier.

For the gene expression analysis, fold difference was calculated using the delta-delta Ct method [24] against the B2M reference gene, and relative to the control groups for the LRT and HRT groups. Data for the *SOST* gene were log base (log10) transformed to achieve a normal distribution prior to analysis. Datasets for genes *Bglap*, *OPG*, *RANKL*, *Runx2*, *SOST* and *Wnt16* were analysed for interactions of phenotype, sex, and training effects on gene expression using a three-way ANOVA. The same analysis was completed for genes *COL1A1*, *COL1A2*, *COL5A1*, *IBSP*, *RANK*, *TGF-β* using a Robust three-way ANOVA test due to the presence of extreme outliers. Pearson correlations or Robust correlations were performed to investigate associations between gene expression and bone collagen synthesis rates (FSR%·d^{-1}) at the tibia mid-shaft. Cook's distance was evaluated for all significant correlations to identify influential data points.

The level of significance was set at $p \leq 0.050$. All data are presented as mean ± 1SD and 95 % confidence intervals [95 % CI]. The effect size generalised eta squared ($\eta 2g$), defined as small ($\eta 2g = 0.01$), medium ($\eta 2g = 0.06$), and large ($\eta 2g = 0.14$) effects, was estimated for all ANOVA analyses. All analyses were performed on RStudio (version 1.4.1717) with packages *tidyverse*, *ggpubr*, *rstatix*, and *WRS2*.

3. Results

3.1. Bone collagen fractional synthetic rates at the femur diaphysis, tibia proximal, tibia mid-shaft, and tibia distal sites

There was a significant large main effect of phenotype at the femur site (Fig. 1; $p < 0.001$; $\eta^2_g = 0.473$), where HRT rats showed greater bone collagen FSRs than LRT rats (HRT 0.178 ± 0.080 [95 % CI 0.152–0.205] %·d^{-1}; LRT 0.079 ± 0.026 [95 % CI 0.051–0.107] %·d^{-1}). There were no other significant interactions or main effects at the femur site.

There was a significant large main effect of phenotype ($p = 0.008$; $\eta^2_g = 0.178$) and a significant large main effect of sex ($p = 0.005$; $\eta^2_g = 0.196$) at the proximal site of the tibia (Fig. 2). Rats from the HRT phenotype showed greater bone collagen FSRs than rats from the LRT phenotype (HRT 0.248 ± 0.136 [95 % CI 0.198–0.298] %·d^{-1}; LRT 0.159 ± 0.091 [95 % CI 0.109–0.209] %·d^{-1}), and male rats also showed greater bone collagen FSRs compared to female rats (males 0.250 ± 0.131 [95 % CI 0.199–0.301] %·d^{-1}; females 0.161 ± 0.101 [95 % CI 0.112–0.210] %·d^{-1}). There were no other significant interactions or main effects at the proximal tibia site.

There was a significant large main effect of training at the mid-shaft site of the tibia (Fig. 3; $p = 0.012$; $\eta^2_g = 0.159$), with rats that underwent interval running training having greater bone collagen FSRs than control rats (trained 0.066 ± 0.055 [95 % CI 0.047–0.085] %·d^{-1}; control 0.039 ± 0.036 [95 % CI 0.017–0.062] %·d^{-1}). There were no other significant interactions or main effects at the mid-shaft tibial site.

Similarly, there was a significant large main effect of training at the distal site of the tibia (Fig. 4; $p = 0.050$; $\eta^2_g = 0.156$), with rats in the

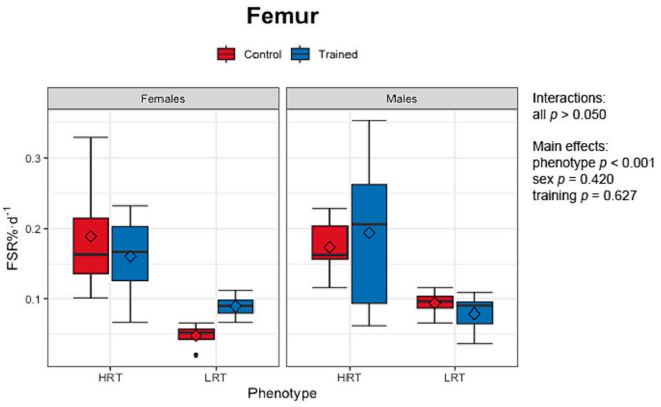

Fig. 1. Three-way ANOVA comparisons of femur diaphysis bone collagen synthesis rates (FSR%·d^{-1}) between phenotypes of high (HRT) and low (LRT) responders to endurance running training, male and female, and trained and control in adult rats. Data shown as raw values (i.e., prior to any transformation). Data are presented as ggplot2 boxplots created with RStudio. Upper and lower whiskers represent the 75th percentile and 25th percentile of the interquartile range, the bolt line represents the median (50th percentile), the diamond represents the mean, and the dot represents an outside value.

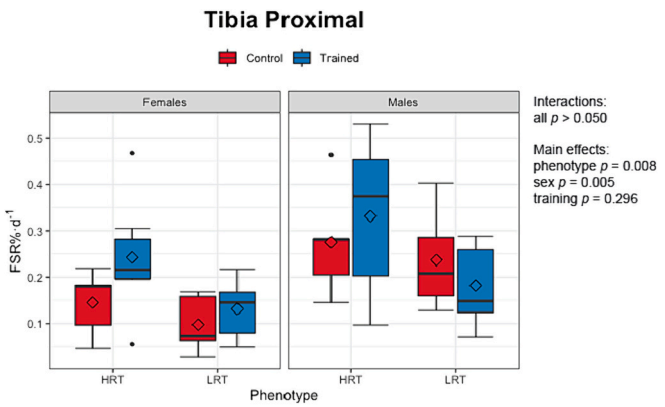

Fig. 2. Three-way ANOVA comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) on the proximal tibia (T-PRO) between phenotypes of high (HRT) and low (LRT) responders to endurance running training, male and female, and trained and control in adult rats. Data are presented as ggplot2 boxplots created with RStudio. Upper and lower whiskers represent the 75th percentile and 25th percentile of the interquartile range, the bolt line represents the median (50th percentile), and the diamond represents the mean.

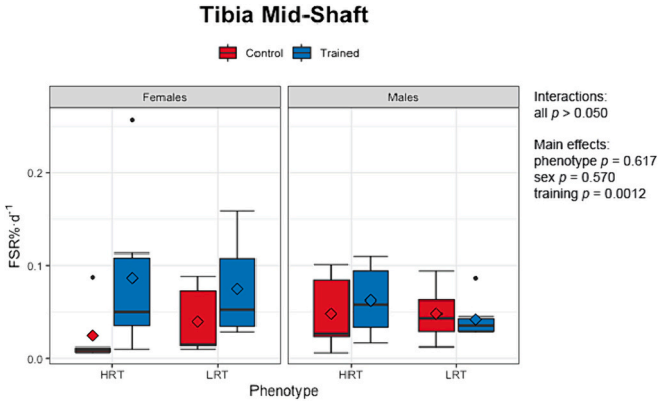

Fig. 3. Three-way ANOVA comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the mid-shaft tibia (T-MID) between phenotypes of high (HRT) and low (LRT) responders to endurance running training, male and female, and trained and control in adult rats. Data shown as raw values (i.e., prior to any transformation). Data are presented as ggplot2 boxplots created with RStudio. Upper and lower whiskers represent the 75th percentile and 25th percentile of the interquartile range, the bolt line represents the median (50th percentile), and the diamond represents the mean.

interval running training group having greater bone collagen FSRs compared to rats in the control group (trained 0.031 ± 0.017 [95 % CI 0.026–0.037] %·d^{-1}; control 0.020 ± 0.010 [95 % CI 0.014–0.027] %·d^{-1}). There were no other significant interactions or main effects at the distal tibia site.

3.2. Gene expression at the tibia diaphysis site

There were no significant phenotype, sex, or interval running training effects on any genes (**Supplementary figures**). Pearson correlations showed significant positive associations between *Bglap* (R = 0.437; p = 0.006), *OPG* (R = 0.538; $p < 0.001$), *RANKL* (R = 0.340; p = 0.034), and *Wnt16* (R = 0.349; p = 0.030) gene expression and bone collagen FSRs at the tibia mid-shaft (Fig. 5). Robust correlations showed significant positive associations between *TGF-β* expression (R = 0.582; $p < 0.001$) and bone collagen FSRs at the tibia mid-shaft, whilst *RANK* expression showed a significant negative correlation with bone collagen FSRs at the mid-shaft tibia (R = −0.459; p = 0.005) (Fig. 5). For the

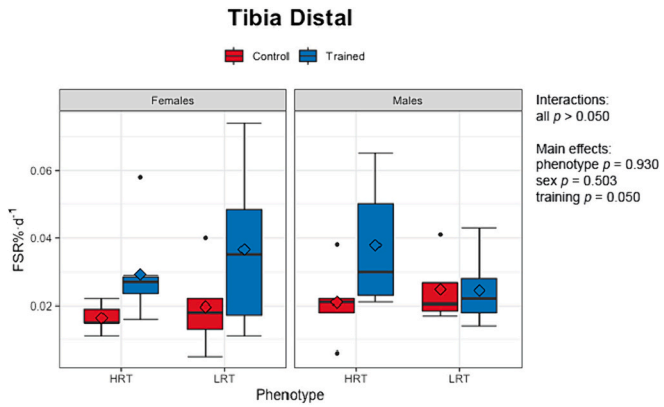

Fig. 4. Robust three-way ANOVA comparisons of bone collagen fractional synthesis rates (FSR%·d^{-1}) at the distal tibia (T-DIS) between phenotypes of high (HRT) and low (LRT) responders to endurance running training, male and female, and trained and control in adult rats. Data shown as raw values (i.e., prior to any transformation). Data are presented as ggplot2 boxplots created with RStudio. Upper and lower whiskers represent the 75th percentile and 25th percentile of the interquartile range, the bolt line represents the median (50th percentile), and the diamond represents the mean.

correlation between *TGF-β* and bone collagen FSRs at the tibia mid-shaft one influential data point (>1.0 Cook's distance) was detected, however, the correlation was still significant after removing this point (R = 0.554; $p < 0.001$).

4. Discussion

In this study the effects of endurance trainability phenotype, sex, and interval running training on bone collagen synthesis in 9-month-old (36 weeks) rats were investigated by using the deuterium oxide stable isotope tracer method that we previously developed [19]. This is the first study to report the interactive effects of these physiological differences on bone collagen synthesis using the direct incorporation of stable isotopes in rat femoral and tibial bones. The effects of these three variables (phenotype, sex, and interval running training) on bone collagen synthesis differed depending upon the site of measurement. Compared to LRT rats, rats from the HRT phenotype showed greater bone collagen synthesis rates at the femur diaphysis and proximal tibia. Interval running training increased bone collagen synthesis rates at the tibial mid-shaft and distal tibia. Male rats had greater bone collagen synthesis rates than female rats, but only at the proximal tibia.

Herein, main effects of phenotype on bone collagen synthesis were reported at the femur diaphysis and proximal tibia; with high responders to endurance training (HRT) showing greater bone collagen FSRs compared to low responders to endurance training (LRT). This evidence indicates that inheriting high trainability for endurance training may induce greater bone formation and/or greater overall bone remodelling independently of sex and interval running training on these two bone sites. In contrast, there were no significant phenotype effects on the mid-shaft and distal tibial sites, which agrees somewhat with a recent study that also measured bone synthesis rates in growing mice (12 weeks old) using a similar D_2O method and reported similar values between inherently high and low active inbred mice (both groups showing bone FSRs of ~0.035 %·d^{-1}), albeit in the skull rather than in the limbs [25]. Interestingly, both the skull and the tibial diaphysis are comprised of predominantly cortical bone, and thus taken together, the results of these two studies suggest that genetic or hereditary factors might influence this type of bone to a lesser extent than trabecular bone (e.g., the predominant type of bone found in the proximal tibia). In support of this assertion, Paternoster et al. [26] showed that cortical and trabecular human bones are affected differently and in a site-specific manner by genetic determinants, and Judex et al. [27] reported more evident

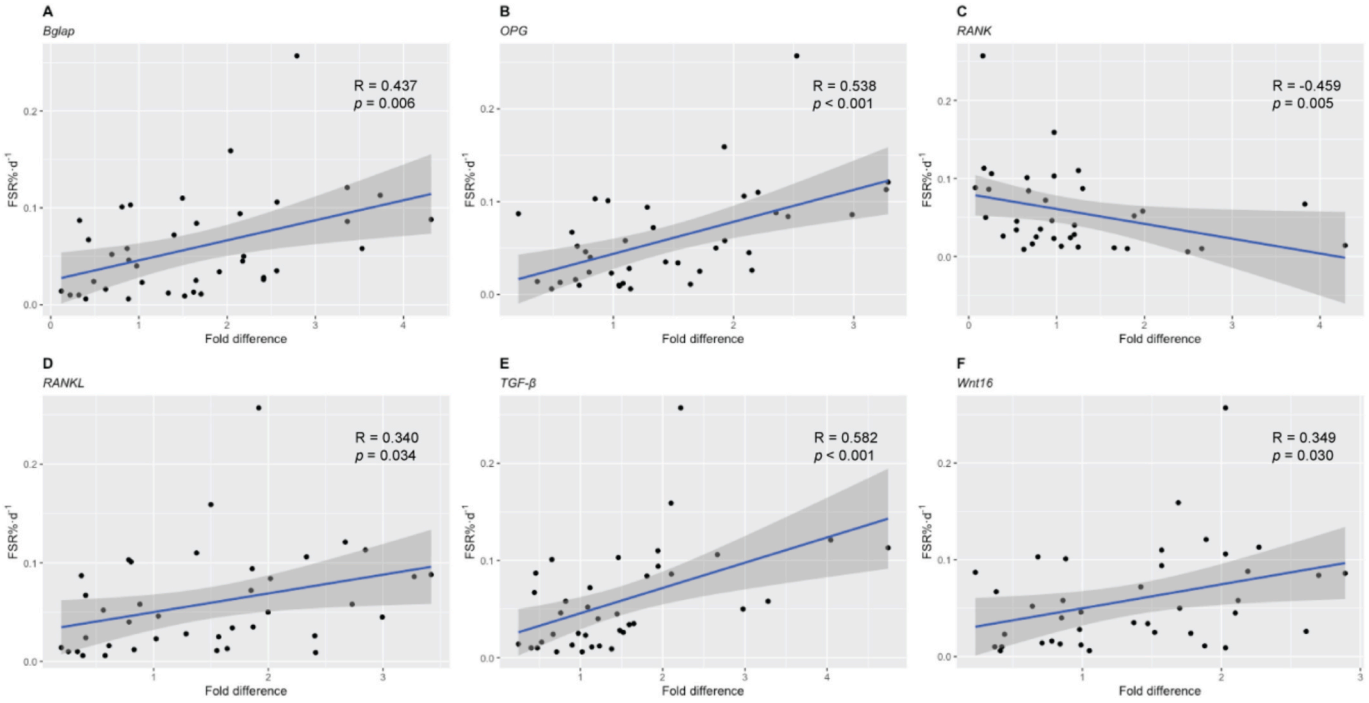

Fig. 5. Pearson (Bglap, OPG, RANKL and Wnt16) and Robust (TGF-β and RANK) correlations between (A) Bglap, (B) OPG, (C) RANK, (D) RANKL, (E) TGF-β, and (F) Wnt16 gene expression (fold difference) and bone collagen synthesis rates (FSR%·d^{-1}) at the tibial mid-shaft (T-MID). Data are presented as ggplot2 scatterplots including regression line created with RStudio.

differences between genetic variations in trabecular regions than in cortical regions in mice. Given that histological analyses were not performed on our bone samples, it is not possible to determine exactly whether the significant effects reported herein denote differences between cortical and trabecular bone.

The effects of HRT and LRT phenotypes on musculoskeletal tissues are not fully understood, with previous studies using the same rat model to investigate skeletal muscle morphology and physiology showing mixed results. Ahtiainen et al. [28] failed to show differences on skeletal muscle morphological characteristics between HRT and LRT rats in control and resistance trained (ladder-climbing) conditions. Two subsequent studies, however, reported impaired skeletal muscle hypertrophy in response to functional overload [29] and exacerbated atrophy after immobilisation [30] in LRT compared to HRT rats. The present study is the first to report HRT and LRT phenotype differences in bone tissue. Further research exploring the phenotype effects on bone and other musculoskeletal tissues with this rat model may help the understanding of musculoskeletal adaptations of highly trained individuals, such as endurance athletes.

Animal and human skeletal sexual dimorphism (e.g., greater bone size and bone mass in males) becomes more prominent with growth and sexual maturation [10]. Herein sex differences on bone collagen synthesis were only evident at the tibial proximal site and not at the distal tibia or the tibial mid-shaft. In addition, there were no sex differences on bone collagen synthesis at the femur. Even though this study was conducted in adult (non-growing) rats, the potential presence of open growth plates in the proximal tibial epiphyses [31] of these rats may explain the greater collagen FSRs in males compared to females at this bone site. The collagen synthesis reported on the proximal tibia may reflect the synthesis of collagen of cartilage from growth plates, indicating greater bone growth in male rats due to the evident sex differences during maturation.

Conversely, research has suggested that animal and human sex-related differences in bone structural adaptations (measured by DXA and pQCT) to exercise/loading exist due to differences in the hormonal environment (e.g., oestrogen) [32]. Whilst some studies have shown blunted responses to running activity at the femur diaphysis in adult female compared to adult male rats [33] and at the tibial diaphysis in mice [34]. These outcomes are not, however, supported by the data presented herein, where, although interval running training positively affected bone collagen synthesis, there was no effect of sex on bone collagen synthesis at the tibial mid-shaft and distal tibia. This fact indicates that sex differences in bone mineral adaptations may not be due to variances in bone formation (by the newly synthesised collagen) across sexes but may be influenced by other factors, such as overall bone remodelling balance.

Bone adaptations to loading are a local phenomenon, as shown in human [12,35] and animal studies [36,37]; these adaptations generally occur on the shaft of long bones, with increases in cortical periosteal apposition [36,38]. Somewhat in agreement, herein, rats completing a 7-week interval running training programme showed greater bone collagen synthesis rates than control rats at the mid-shaft and distal sites of the tibia, which suggests that the training intervention had an osteogenic effect by increasing formation of newly synthesised bone matrix at these two sites. Despite high variability in bone collagen FSRs, these effects were statistically large for this sample. Evidence from the mineral adaptations of bone have shown similar outcomes. A study in young growing female rats (3 weeks old) showed increased BMC (measured by DXA) at the middle and distal tibial sites, but not at the proximal tibia after a 7-week exercise intervention, where the rats ran on a treadmill [39]. These site-specific osteogenic effects from running may be due to differences in cortical and trabecular bone adaptations to loading [40]. Indeed, a recent study in female mice (14 weeks old), which applied controlled mechanical loading for 4 weeks, reported small or no effects on trabecular bone (at the proximal tibia site) and greater effects on cortical bone (at the tibia diaphysis), particularly at the periosteal surface [41]. In comparison, in humans (women, 24 ± 2 years), 44 weeks of heavy miliary training resulted in positive adaptations in trabecular and cortical bone at the tibial metaphysis and, in line with the previous observations, only in the cortical compartment in the tibial diaphysis [42]. Differences in loading magnitude may also be reflected in bone adaptations; although these are usually studied using artificial loading

interventions, which have reported higher loading magnitudes in the proximal tibia and lower magnitudes in the more distal tibial sites [36,43]. Herein, the differences in training load could have affected bone collagen synthesis rates, as the running speed was adjusted to each individual rat. Due to the lack of methods for measuring loads across bone sites during in vivo exercise [44], however, the site-specific loading characteristics of running and if/how this factor may have influenced the results reported herein, remain unknown.

Although interval running training effects in bone collagen synthesis rates at the tibia mid-shaft did not translate into significant changes in gene expression of collagen genes at the tibial diaphysis, osteocalcin (*Bglap*), *TGF-β*, *OPG*, and *Wnt16* gene expression was positively correlated with tibial mid-shaft bone collagen synthesis. This outcome suggests that these four genes may be a key regulatory factor for osteogenic responses to exercise and subsequent bone formation. In fact, expression of *Bglap*, *TGF-β*, *OPG*, and *Wnt16* has previously been associated with mechanical loading stimulation [45–48] and/or bone formation [49–52]. Interestingly, expression of *RANK*, which promotes osteoclast differentiation and thereby bone resorption [53], was negatively correlated with bone collagen FSRs at the tibial mid-shaft. In support of this result, recent evidence has shown that *RANK* silencing promotes osteoblast differentiation and bone formation in vitro [54,55], indicating that downregulation of RANK may be required for increased bone formation.

4.1. Strengths and limitations

The principal strength of the study is the application of a stable isotope tracer methodology for the measurement of physiological differences on bone collagen formation in rodent loaded long-bones, including the reporting of greater bone formation in highly cortical bone sites in response to a 7-week running-exercise intervention. The study also includes some limitations such as the potential presence of growth plates in the proximal tibia site, the differences in training load within the trained groups, and the small sample sizes in each comparison group, all of which could have contributed to larger variability in the results.

4.2. Conclusions and future directions

Bone collagen synthesis reflects the formation of newly synthesised bone matrix during the bone (re)modelling cycle. The different physiological effects (response to endurance trainability phenotype, sex and interval running training) on bone collagen synthesis shown at four bone sites (femur diaphysis, proximal tibia, tibial mid-shaft and distal tibia) indicate that these variables affect bone formation in a site-specific manner. This evidence further emphasises the importance of investigating heritability, sex, and lifestyle effects on bone (re)modelling on a site-specific basis and the limitations of using whole-body indicators (e.g., whole-body BMD, circulating bone markers). Whilst non-modifiable factors had effects on rats bone collagen synthesis at the femur diaphysis (phenotype) and proximal tibia (phenotype and sex), interval running training had a positive effect on bone collagen synthesis at the tibial mid-shaft and distal sites.

Further animal and human research combining stable isotope techniques and histology would provide further insight into physiological effects on bone collagen synthesis on trabecular and cortical bone sites. Studies focusing on the bone adaptations to exercise should explore how other types of exercise regimens (e.g., shorter/longer interventions, shorter bouts of running or jumping) affect bone formation and collagen synthesis at different sites. It remains unknown whether site-specific bone collagen synthesis changes are linked to changes on the bone mineral structure at the same bone sites.

CRediT authorship contribution statement

Rita Civil: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matthew S. Brook:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lívia Santos:** Writing – review & editing, Supervision, Conceptualization. **Ian Varley:** Writing – review & editing, Supervision, Conceptualization. **Kirsty J. Elliott-Sale:** Writing – review & editing, Supervision, Conceptualization. **Sanna Lensu:** Writing – review & editing, Resources, Methodology, Investigation. **Juha P. Ahtiainen:** Writing – review & editing, Resources, Methodology, Investigation. **Heikki Kainulainen:** Writing – review & editing, Resources, Methodology, Investigation. **Lauren G. Koch:** Writing – review & editing, Resources, Methodology, Investigation. **Steven L. Britton:** Writing – review & editing, Resources, Methodology, Investigation. **Daniel J. Wilkinson:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Kenneth Smith:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Philip J. Atherton:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization. **Craig Sale:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2024.117257.

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